ORIGINAL ARTICLE

Dillenia suffruticosa Dichloromethane Root Extract Reduced Metastasis of 4T1 Cells to the Liver and Heart Without Causing Toxicity in Female BALB/C Mice

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ABSTRACT

Introduction: Breast cancer is ranked first among other cancers in women. Ineffectiveness of current treatments and adverse effects such as multiple organ failure and nephrotoxicity are the common problems faced in cancer therapy. Therefore, alternatives to treat breast cancer metastasis with fewer toxic effects are actively sought-after. *Dillenia suffruticosa* (DS) commonly known as 'Simpoh air' has been a traditional remedy for cancer growth. Therefore, this study investigated the metastasis inhibiting properties of DS root dichloromethane extract (DCMDS) in tumour bearing female BALB/c mice and sub-acute multiple dose oral toxicity upon treatment with this extract. **Methods:** Forty-eight tumour bearing mice were given either oral treatment of DCMDS (50, 100 and 200 mg/kg) or doxorubicin (2 mg/kg) for 28 days and the degree of metastasis was analysed in each group. Thirty other female BALB/c mice were treated with DCMDS (50, 100 and 200 mg/kg) and the general behaviours, biochemical, haematological and histopathological changes were observed. Data were analysed with One-way ANOVA and Dunnet's test where $p<0.05$ was considered significant. **Results:** All doses of DCMDS showed lowered metastatic cells in liver and DCMDS at (50 and 100 mg/kg) had less metastatic cells in the heart compared to doxorubicin (2 mg/kg). All DCMDS treated groups showed no abnormal behaviours and all tested physiological parameter values fall within the normal ranges. **Conclusion:** DCMDS reduced metastasis of 4T1 cells to the liver and heart better than doxorubicin without causing toxicity. This study highlights that DCMDS is a promising drug to be further developed for cancer therapy.

Keywords: *Dillenia suffruticosa*, Breast cancer, Metastasis, Toxicity

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INTRODUCTION

Breast cancer ranked the first among other cancers in women and the incidence is increasing dramatically worldwide. There are several standard treatments for patients with breast cancer including radiotherapy, chemotherapy, hormone therapy and surgery. Nevertheless, they are not effective especially to treat breast cancer metastasis (1). Moreover, these treatments come with several adverse effects including organ failure, immune-suppression and development of drug resistance (2-3).

Doxorubicin is the frontline drug used since 1974 for cancer treatment. The Food and Drug Administration has approved doxorubicin as one of the most potent chemotherapeutic drugs (4). However, though it provides cure in some cancer cases, its usage is limited due to its toxic effect on non-cancerous cells affecting major organs, and could cause cardiotoxicity which is life-threatening. Therefore, treatment with doxorubicin is only applicable in selected cases (5).

Failure to subsequent therapy and relapse of rapid tumour growth are caused by the emergence of tumour cells that are resistant to anticancer drugs and other types of cancer treatment (6-8). Hence, there are lots of efforts in finding alternative for management and treatment of breast cancer. Currently, researchers have started to search for new alternative of anticancer drugs

derived from plants, marine and microorganism (9). It is believed that naturally derived anticancer drugs are safe (10). However, the necessity for toxicity study of natural products increases as there are reports of toxic effects upon its consumptions.

Dillenia suffruticosa (DS) is one of the potential herbs with various pharmacological properties. It is locally known as 'Simpoh air' that can be found in West Malaysia, Philippines, Indonesia and Brunei. The fruit pulp has been traditionally used as treatment for headache and as a wound healing agent (11). The leaves are used as traditional remedies to treat microbial and fungal infection (12) and the fruit as a treatment for cancerous growth (13).

The methanol root extract of DS exhibited antioxidant activities and cytotoxicity towards several cancer cell lines especially HeLa. The dichloromethane and ethyl acetate extract of DS exhibited strong cytotoxicity towards selected cancer cell lines such as HeLa, MCF-7, A549, MDA-MB-231 and HT29 by initiating apoptosis and cell cycle arrest (14). The phytochemical studies suggest the constituents of saponins, terpenoids, sterols and polyphenolic compounds that contribute to the cytotoxic properties (15). DS root dichloromethane extract (DCMDS) exhibited cytotoxicity, induced cell cycle arrest and apoptosis towards MCF-7 and MDA-MB-231 breast cancer cell lines (16-17). DCMDS exhibited the potential of targeting cancer cells with mutant caspase-3 (MCF-7) by induction of cell cycle arrest and apoptosis via multiple signaling pathways (16). Betulinic acid was reported to be the main cytotoxic compound in DCMDS (17). Betulinic acid has various polypharmacological activities that include antiretroviral, antimalarial, anti-inflammatory and anti-cancer (18-22). The aqueous extract of DS root possessed anti-breast cancer properties (23) and might be an effective therapy to treat this condition.

Even though the anti-breast cancer properties of DCMDS have been reported, its ability to inhibit cancer metastasis to other adjacent organs remains unexplored. Since recently used anti-cancer therapy are companied with complications such as multiple organ failure (24) and nephrotoxicity (25), it is important to outline the safety margin of this drug ensure that it is not potentially harmful to the host. Therefore, this study determined the metastasis inhibition property of DS root dichloromethane extract (DCMDS) in tumour bearing female BALB/c mice and the possible sub-acute multiple dose oral toxicity of DCMDS.

MATERIALS AND METHODS

Dillenia suffruticosa

The fine powder of root of DS was supplied by Primer Herber Sdn. Bhd., Malaysia. Identification and authentication of the plant was done at the Biodiversity Unit, Institute of Bioscience, UPM (Voucher specimen number SK 1937/11).

Preparation of DCMDS

Briefly, 150 g of fine powder of the root of DS was macerated with dichloromethane. Then, the mixture was filtered using Whatman filter paper No. 1. Residues of the mixture were re-extracted with dichloromethane three times. The filtrates were pooled and dichloromethane was eliminated using a rotary evaporator at 40°C. The extract was weighed and stored at -20°C. The percentage of yield was then calculated using the following formula:

$$\text{Yield (\%)} = \frac{\text{(Weight of crude extract)}}{\text{(Weight of dried plant materials)}} \times 100$$

Cell culture

The 4T1 mouse mammary carcinoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% of antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). The cells were maintained in a humidified atmosphere at 37°C containing 5% of carbon dioxide.

Experimental animal

The protocol of the study was reviewed and approved by the Institutional of Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM/IACUC/AUP-R068/2015). Female 6-8 weeks old BALB/c mice with 20–30 g in weight were used in this study. They were housed in individual cages with a period of 12-h light and 12-h dark cycle, in a room with temperature set at 20–24 C with 40%–50% relative humidity. All the mice were acclimatized for at least a week before starting the experiment. The mice were fed with a standard food pellet (Gold Coin, Malaysia) and were provided with tap water ad libitum.

Experimental design

In vivo anti-breast cancer study of DCMDS

Forty-eight female BALB/c mice were allocated into six groups (n=8), which were negative control (with breast cancer, untreated), positive control (with breast cancer, treated with doxorubicin 2 mg/kg), normal control (without breast cancer, untreated) and another three groups of mice with breast cancer, treated with 50 mg/kg, 100 mg/kg and 200 mg/kg of DCMDS, respectively, except for the normal control. All mice were injected with 4T1 cells (1 x 105 4T1 cells/0.1 mL of PBS) into the mammary fat pad. When the tumour was palpable, DCMDS was administered orally to the animals via gavage for 28 days.

The mice were weighed three times a week. The tumour volume was measured twice weekly by using a digital vernier caliper. The following formula was used to determine the volume of the tumours (26):

$$\text{Volume of the tumours} = \text{Length} \times \text{width}^2 \times 0.52$$

Mortality of the mice was recorded to calculate the survival time. The major organs (heart, kidneys, spleen, liver, lungs) were excised for fats, weighed and subjected to gross observation. The organ- to- body weight ratio of each mouse was calculated.

Sub-acute toxicity study of DCMDS

Thirty female BALB/c mice were assigned in a random way into five groups (n=6), which were the normal and negative control, and three treatment groups of escalating dose of DCMDS (50, 100 and 200 mg/kg). The doses were selected based on solubility test and acute toxicity study. The negative control group received 10% DMSO. For the treatment groups, DCMDS was dissolved in 10% DMSO and orally administered daily for 28 days. The animals were monitored twice daily (before and after dosing) for general health such as general appearance, behaviour, toxicity symptoms and mortality in 28 days. The body weight was measured at a time interval of three days (Day 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28).

Biochemical and haematological analysis

Blood collection was carried out on Day 29 in the sub-acute toxicity study. Prior to blood collection, the mice were anesthetized under ketamine (75 mg/kg; ip) and xylazine (5 mg/kg; ip). The blood samples (1 mL) were collected by cardiac puncture by using a 26 G x 1/2 needle" (Terumo®, Belgium) and centrifuged at 10000 rpm for 10 minutes to separate the serum. Blood biochemical and haematological analysis were carried out by using a chemistry analyser (Selectra XL, Dieren, Netherlands) and hematoanalyser, respectively. For liver hepatic function, level of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatise (ALP) was evaluated. For kidney renal function, blood urea nitrogen and serum creatinine level was determined.

Histopathological observation

Following blood sampling, the mice were sacrificed. Tumours and organs including heart, lungs, kidneys, liver and spleen were harvested and placed in 10% formalin fixative. After fixation for at least 24 hours, the tissues were processed for 16 hours by an automated tissue processor (Leica ASP6025, Leica Microsystems, Wetzlar, Germany). The processed tissues were then embedded into paraffin blocks. Five micrometres thick sections were cut and stained with haematoxylin and eosin (H&E). Each slide was examined under a light microscope with guidance of a pathologist. The histological changes were evaluated by from 10 random fields from each slide of each group.

For the sub-acute, multiple dose toxicity study, the scoring system for histological changes of liver and kidney was based on degree of necrosis of hepatocytes, chromatin condensation, light cytoplasm, nucleus fragmentation, and sinusoidal widening in liver while degree of dilation in the glomeruli and tubular necrosis in kidney (27).

For the anti-breast cancer study, the slides were viewed for any metastatic cell and any organ that had one metastatic cell, and later recorded as "with metastasis".

Statistical data analysis

The data were analysed by Graphpad prism 5.0. One way ANOVA analyses were performed followed by Dunnet's test. The data were presented as mean ± SEM. Value of $p<0.05$ was considered as significant.

RESULTS

Anti-breast cancer properties of DCMDS

Effects of DCMDS on the body weight and relative organ weight in tumour-bearing female BALB/c mice

Body weight changes and relative organ weight of tumour-bearing female BALB/c mice are shown in Fig. 1A and 1B, respectively. There were no significant fluctuations in the body weight and relative organ weight of all the DCMDS-treated groups compared to the normal control group ($p>0.05$).

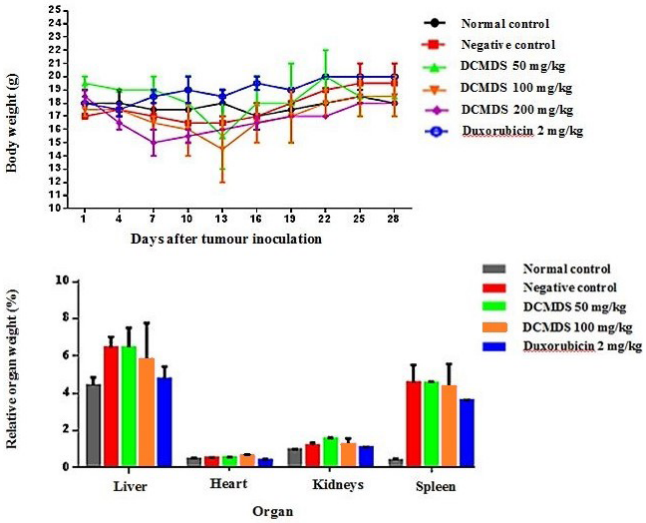

Figure 1: (A) Body weight (B) Relative organ weight of 4T1 tumour-bearing female BALB/c mice treated with DCMDS for 28 days. Each column represents the mean ± S.E.M. of six mice. *$p<0.05$ compared with the control group (one-way Anova followed by Dunnett's test).

Effects of DCMDS on the tumour volume and survival rate in tumour-bearing female BALB/c mice

Tumour volume and survival rate of tumour-bearing female BALB/c mice are shown in Fig. 2 and 3, respectively. There were no significant changes in the tumour volume of all the DCMDS-treated groups compared to the negative and positive control groups ($p>0.05$). There was no apparent difference in the survival prolongation between the DCMDS-treated groups and both the control groups (log-rank test, $p>0.05$).

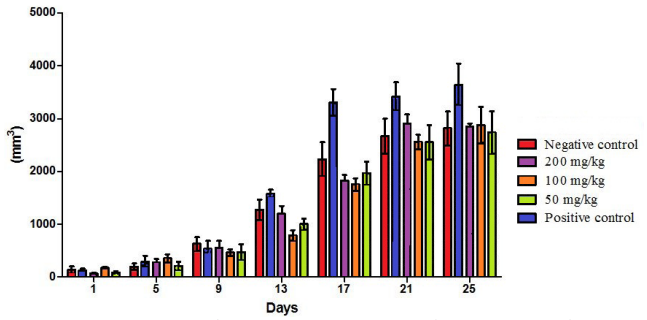

Figure 2: Tumour volume of 4T1 tumour-bearing female BALB/c mice treated with DCMDS for 28 days. Each column represents the mean ± S.E.M. of six mice. *p<0.05 compared with the control group (one-way Anova followed by Dunnett's test).

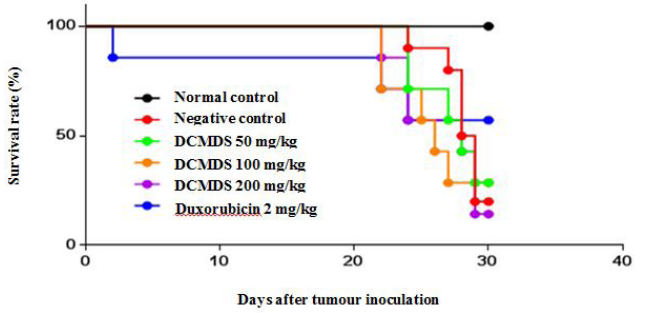

Figure 3: Survival rate of 4T1 tumour-bearing female BALB/c mice treated with DCMDS for 28 days.

Effects of DCMDS on metastasis in tumour-bearing female BALB/c mice

Lungs of the tumour-bearing female BALB/c mice given DCMDS treatment are shown in Fig. 4. The group treated with doxorubicin has the least number of tumour nodules. Less number of tumour nodules was noted in the treatment with 200 mg/kg of DCMDS compared to 50 and 100 mg/kg DCMDS-treated groups. Fig. 5 shows the presence of metastatic cells that appeared in clusters in the liver and heart sections of 4T1- tumour-bearing female BALB/c mice. All doses of DCMDS showed lowered metastatic cells in liver and DCMDS at (50 and 100 mg/kg) had less metastatic cells in the heart compared to doxorubicin (2 mg/kg).

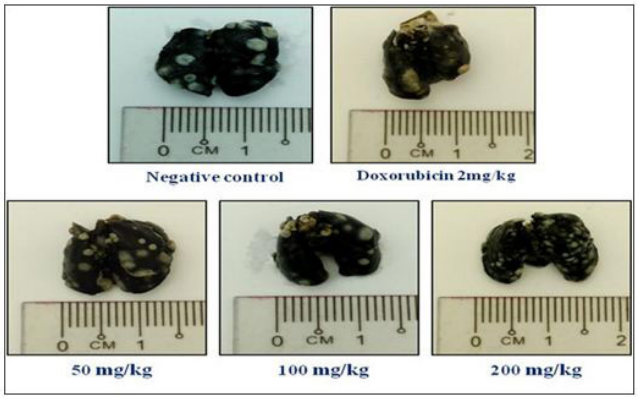

Figure 4: Lungs of 4T1 tumour-bearing female BALB/c mice treated with DCMDS for 28 days. The tumour nodules (white spots) are metastasized from breast cancer cells.

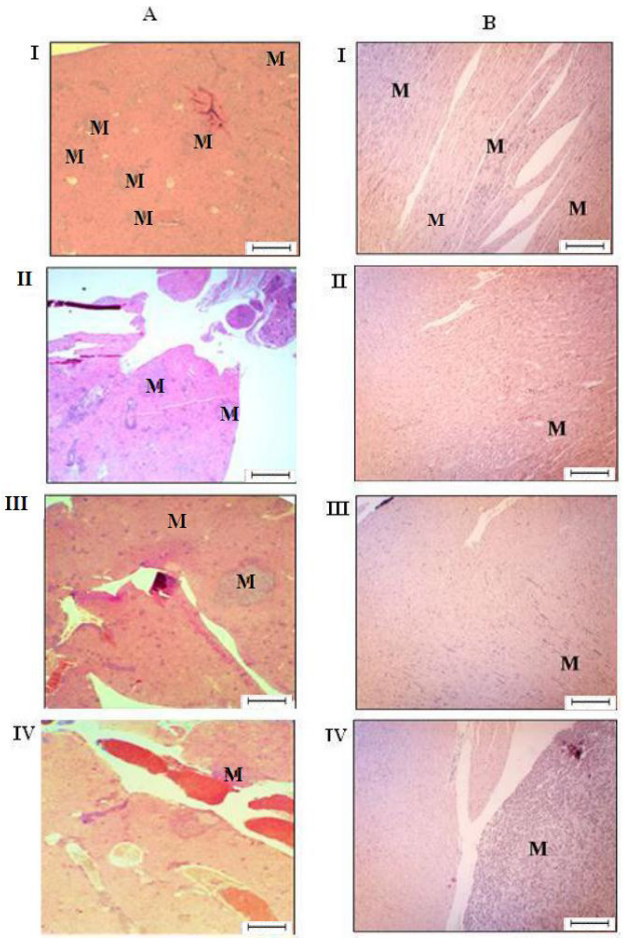

Figure 5: (A) Liver (B) Heart sections of 4T1-tumor-bearing female BALB/c mice treated with (I) doxorubicin 2 mg/kg, (II) DCMDS 50 mg/kg, (III) DCMDS 100 mg/kg, (IV) DCMDS 200 mg/kg as observed under a light microscope after staining with hematoxylin and eosin. The livers and hearts of 4T1 tumour-bearing mice showed infiltration of neoplastic cells. The cells were poorly differentiated and characterized by the presence of large hyperchromatic nuclei and small amount of cytoplasm. Metastatic cells that appeared in cluster are marked by M (40X magnification). Scale bar represents 100 µm.

Sub-acute multiple dose oral toxicity of DCMDS

Effects of DCMDS on the body weight and relative organ weight of female BALB/c mice

Changes in the mean body weights values of female BALB/c mice treated with DCMDS for 28 days are illustrated in Fig. 6A. There was no significant difference in the body weight of all the DCMDS-treated groups compared to the normal control group ($p>0.05$) throughout the experimental period. Fig. 6B illustrates various organs to body weight ratio (kidneys, liver, spleen, heart and lungs) of female BALB/c mice treated with DCMDS. There was no significant difference in the organs to body weight ratio of all the DCMDS-treated groups compared to the normal control group ($p>0.05$) throughout the experimental period.

Effects of DCMDS on the biochemical parameters and haematological profile in female BALB/c mice

Level of liver enzymes, kidney functions and complete blood profile of female BALB/c mice given DCMDS

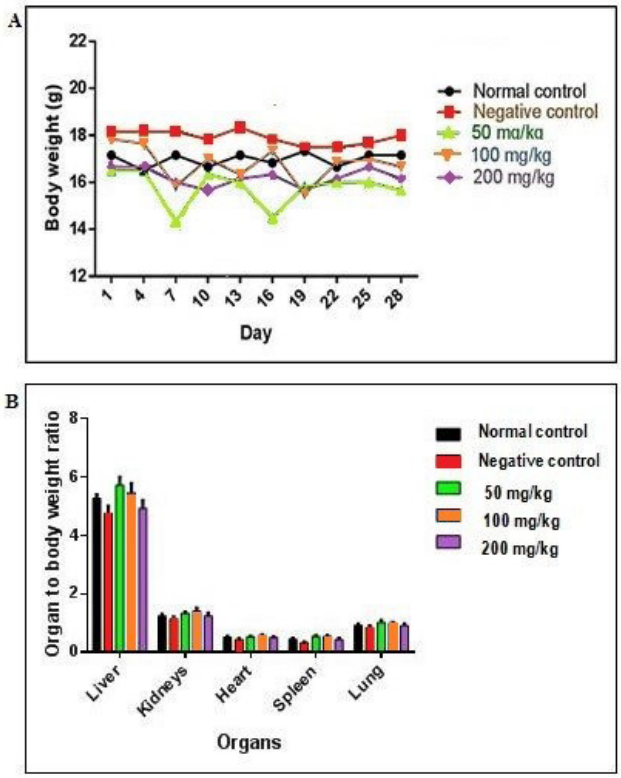

Figure 6: (A) Body weight (B) Organ to body weight ratio of female BALB/c mice treated with DCMDS in the sub-acute toxicity study for 28 days. *p<0.05 compared with the control group (one-way Anova followed by Dunnett's test).

treatment is shown in Table I. There was a significant decrease in the level of ALP in the group of 50 and 200 mg/kg of DCMDS and negative control compared to the normal control group ($p<0.05$). There were no significant changes in the complete blood profile of all the DCMDS-treated groups compared to the normal control group ($p>0.05$).

DISCUSSION

Since currently available anti-cancer agents exert undesired health issues, the potential of DCMDS as anti-breast cancer agent has been investigated alongside the possible toxic effect of this extract. The anti-breast cancer and cancer metastasis properties of DCMDS were determined using the 4T1 tumour-bearing BALB/c mice model. The model worked whereby the cancer developed after seven days of induction. In addition, it was also evidenced by the higher relative spleen weight of the 4T1 tumour-bearing mice compared to the normal mice. The spleen harvested from the 4T1 tumour-bearing mice showed sign of splenomegaly (the enlargement of the spleen)(data not shown), which is the change in splenic architecture due to the presence of tumour that increased the circulating neutrophils and other leukocytes (mature blood cells) and the occurrence of extramedullary hematopoiesis (28).

The weight of the tumour-bearing mice in all groups increased after 2 weeks of induction except for the positive control group (2 mg/kg doxorubicin) probably due to the side effects of the drug that causes reduced appetite, hence reduced body weight. Even though insignificant, a reducing trend in the body weight was noted starting from day 4 until day 13 for the negative control, 50 and 100 mg/kg of DCMDS-treated groups, and from day 1 until day 7 for 200 mg/kg of DCMDS-treated group. The reason behind the condition remains unclear. UK Home Office Regulation reported that weight loss is expected in tumour-bearing mice after 4 weeks of breast cancer induction and the mice could lose up to 25% of their body weight (29). Nevertheless, in this study, the weight loss occurred as early as 7 days after induction. It is believed due to the use of higher concentration of 4T1 cells i.e. 5×10^5 / 0.1 mL cold PBS as compared to 1×10^5 / 0.1 mL cold PBS (29) that causes the cancer to be developed faster and more aggressive. This perhaps will bring more pain that will lead to stress, which will suppress the appetite. Loss of appetite will make the animals to consume less food and therefore reduce the body weight. The animals also presented some features of cancer-anorexia cachexia syndrome or defined as wasting syndrome, which involves muscle mass and fat loss directly instigated by tumour factors or indirectly by an unusual response by host to the presence of tumour (30).

In this experiment, doxorubicin was used as a positive control. Doxorubicin is one of the chemotherapeutic drugs used to treat metastatic breast cancer. The complex mechanism of action of doxorubicin includes blocking of topoisomerase II production and induction of DNA double-strand breaking, interference in the unwinding of DNA, initiation of differentiation, and production of reactive oxygen species (31). Among all the treatment groups, doxorubicin at 2 mg/kg was found to be the most potent only for inhibition of metastasis to the lungs, the first organ that the cancer cells will spread to due to its near distance to the breast. This is proven by the least number of tumour nodules (metastatic cells) detected in the lungs based on the macroscopic observation as compared to other groups. The other reason of doxorubicin giving the best effect is probably due to the route of administration, which is via intraperitoneal injection. Absorption of substance delivered intra-peritoneally (IP) is usually much slower than for intra-venous (IV) injection but it is delivered faster compared to oral administration (32). Even though the absorption of the delivered material is faster for IP injection as compared to oral administration, the pharmacokinetics of substances in both IP and oral administration are the same due to the main route of absorption is via mesenteric vessels, which is moved into the portal vein and pass through the liver (33). Thus, it is believed that IP administration of doxorubicin helps to give the good effect of the drug delivery to targeted sites (breast). Data of this in vivo study further support the antibreast cancer properties of DCMDS towards MCF-7 and MDA-MB-231 breast cancer cell lines (16-17). DCMDS is therefore a very potent option to be

further explored as breast cancer therapy.

The possible toxic effect that might be exerted by DCMDS was evaluated from sub-acute multiple dose toxicity study where female BALB/c mice were on repeated oral administration of DCMDS for 28 days. The subacute oral toxicity serves as an essential test for assessing safety (34). An ingested item will be transported to the liver for detoxification and kidney for urea removal by the blood. Therefore a high toxic level would be indicated in the blood and the detrimental effect of the drug will affect the function of those organs. The subacute multiple dose oral toxicity screening for the blood profile, organ function and histopathological effect of the organs will be able to provide us with the necessary information on the toxic effect of DCMDS. There were no deaths due to the employed treatment and signs of morbidity at any dose level of DCMDS tested (50, 100 and 200 mg/kg). All the animals were absent from any abnormal symptoms such as restlessness, unresponsiveness and failure to groom. General performance or activity and body weight are one of the important parameters for evaluation of first signs of toxicity (35).

In addition, the treated animals showed no significant weight loss throughout the experiment, suggesting that they are free from any adverse effects of DCMDS. Changes in the body weight of an animal are measure of an unfavourable effect of a drug or the chemical to be tested (36). The DCMDS treatment groups were having percentage of organ to body weight ratio of kidneys, spleen, liver, heart and lungs similar to the normal control suggesting that DCMDS does not injure or affect any organ development of the animals. This is supported by (37), whereby relative organ weight is one of the indications to detect if the organs have been attained some form of injury.

The plasma level of liver enzymes (ALT and AST) of DCMDS-treated groups was in the normal range. Liver is the main organ associated with the biotransformation of drugs (38). Liver is very prone to xenobiotic-induced injury due to its principal role in metabolism of xenobiotic (39). Biomarkers of the serum levels of liver enzymes are able to represent liver health. As mentioned by (40), hepatotoxin and drugs that are toxic to the liver causes a hike in the level of serum AST, ALT and ALP. ALT specifically detects liver health and therefore is a better parameter to identify liver injury. Possible disease to muscles and heart could cause changes to AST levels (41). ALT, a cytoplasmic enzyme is present at high levels in the liver and a rise in the concentration of this enzyme indicates hepatocellular damage (42). In contrast to ALT, ALP is found commonly at the cell lining of bile duct in the liver and is useful in the detection of any blockade to the biliary system (43). Even though there was a significant drop in the level of ALP in the groups treated with 50 and 100 mg/kg of DCMDS, the histopathological observation revealed no abnormalities in the liver.

There was no significant difference in both urea and creatinine serum levels in all of the DCMDS-treated groups compared to the normal control group suggesting that the extract does not cause any toxicity to the kidney and alter the renal functions. Urea and creatinine levels could effectively indicate kidney dysfunction (44) and the elevation in the blood creatinine indicates a reduced kidney function (45). Creatinine is the product of the catabolism of phosphate in skeletal muscle and will increase when renal function is poor (46). As for the serum urea, it can increase due to the toxic effect on renal parenchyma, renal tubules and a possible block in the urinary outflow tract (47).

Treatment with DCMDS also did not affect the haematological profile of the animals including red blood cells, white blood cells, haemoglobin, packed cell volume, mean corpuscular haemoglobin, mean corpuscular volume, platelet and plasma protein. According to (44), hematopoietic system, a sensitive target for compounds that are toxic serves as a vital index of its functional and pathological status. It is suggested that DCMDS does not affect the physiological function of the animals. As suggested by (48), blood parameters analysis is appropriate for evaluation of risk of toxicity, as alterations to the haematological systems could highly predict human toxicity from the outcome of animal study is translated to human use.

From the histopathological observation, the liver tissue from all the DCMDS-treated groups did not show any sign of abnormalities such as necrosis, inflammatory cell infiltration and haemorrhage, similar to the normal control. It is suggested that DCMDS is not toxic to the liver, in agreement with the data on level of serum ALT, AST and ALP. This is supported by (37) that the impaired organs often have abnormal atrophy. Gross examinations after post-mortem dissection of the animals further confirmed that DCMDS did not cause any organ damage as all the organs are normal in size and have similar structural architecture with the normal control. As for the kidney tissue, there was also absence of any histological changes or abnormalities such as necrosis, inflammatory cell infiltration and haemorrhage in all the DCMDS-treated groups, similar to the normal control group, which indicates the absence of toxicity. The gross examinations of kidneys also revealed no abnormality, and the relative organ weight of kidneys was indifferent compared to the control. It is suggested that DCMDS is not toxic to the kidneys, in agreement with the data on level of serum creatinine and urea.

Considering that DCMDS showed lower cancer metastasis compared to doxorubicin and recorded no toxic effect to the vital organs such as kidney and liver, it is a potential candidate for the treatment of breast cancer therapy.

CONCLUSION

DCMDS has reduced the severity of metastasis to the liver and heart of 4T1 tumour-bearing female BALB/c mice better than doxorubicin. Based on the normal behaviour, body weight, organ to body weight ratio, biochemical profile, haematological profile and organ histology from the sub-acute multiple dose oral toxicity study, the treatment with 50, 100 and 200 mg/kg DCMDS was not toxic to the animals. DCMDS also was considered safe when given orally to the animals in repeated dose. DCMDS is therefore a highly potential agent to be established for breast cancer therapy. However, more research is necessary to further elucidate the exact mechanism of action of this drug and its therapeutic value in other types of cancer. Furthermore, its effect should also be compared with other currently available anti-cancer drugs to evaluate if DCMDS is a promising substitute to the present cancer therapy.

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